MOLECULAR AND CELLULAR BIOLOGY, July 2001, p. 4604–4613 Vol. 21, No. 14
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.14.4604–4613.2001

Characterization of Mediator Complexes from HeLa Cell Nuclear Extract

GANG WANG, GREG T. CANTIN, JENNITTE L. STEVENS, AND ARNOLD J. BERK*

Molecular Biology Institute, University of California, Los Angeles, California 90095-1570

Received 11 January 2001/Returned for modification 24 February 2001/Accepted 18 April 2001

A number of mammalian multiprotein complexes containing homologs of *Saccharomyces cerevisiae* Mediator subunits have been described recently. High-molecular-mass complexes (1 to 2 MDa) sharing several subunits but apparently differing in others include the TRAP/SMCC, NAT, DRIP, ARC, and human Mediator complexes. Smaller multiprotein complexes (~500 to 700 kDa), including the murine Mediator, CRSP, and PC2, have also been described that contain subsets of subunits of the larger complexes. To evaluate whether these different multiprotein complexes exist in vivo in a single form or in multiple different forms, HeLa cell nuclear extract was directly resolved over a Superose 6 gel filtration column. Immunoblotting of column fractions using antisera specific for several Mediator subunits revealed one major size class of high-molecular-mass (~2-MDa) complexes containing multiple mammalian Mediator subunits. No peak was apparent at ~500 to 700 kDa, indicating that either the smaller complexes reported are much less abundant than the higher-molecular-mass complexes or they are subcomplexes generated by dissociation of larger complexes during purification. Quantitative immunoblotting indicated that there are about 3×10^5 to 6×10^5 molecules of hSur2 Mediator subunit per HeLa cell, i.e., the same order of magnitude as RNA polymerase II and general transcription factors. Immunoprecipitation of the ~2-MDa fraction with anti-Cdk8 antibody indicated that at least two classes of Mediator complexes occur, one containing CDK8 and cyclin C and one lacking this CDK-cyclin pair. The ~2-MDa complexes stimulated activated transcription in vitro, whereas a 150-kDa fraction containing a subset of Mediator subunits inhibited activated transcription.

The *Saccharomyces cerevisiae* Mediator complex was originally identified because of its ability to stimulate activated transcription in vitro. Many of the subunits of the purified Mediator complex (24) are encoded by *SRB* genes, first characterized as suppressors of a deletion in the C-terminal heptapeptide repeat (CTD) of the large subunit of RNA polymerase II (Pol II) (23). Additional Mediator subunits are encoded by genes initially identified in other genetic screens for mutations affecting gene control and are named accordingly (e.g., *RGR-1*). Mediator subunits not characterized previously were called Med1, Med2, etc. (24). Mediator subunits in yeast have also been purified as part of a still larger holoenzyme complex including Pol II and several general transcription factors (GTFs) (23). The Pol II holoenzyme analyzed by Young and colleagues (see reference 23 for a review) includes Srb8, Srb9, Srb10, and Srb11, whereas the Mediator complex studied by Myers et al. (24) lacks these subunits. The Srb8 to Srb11 subunits form a functional subcomplex of the holoenzyme required for repression by several yeast repressors (3, 11, 23). These subunits are regulated differently from other yeast Mediator and holoenzyme subunits. The intracellular Srb10 concentration falls dramatically as yeast cells deplete nutrients from their media, whereas the concentrations of other Mediator subunits do not (11). Recently, Liu et al. analyzed yeast Mediator complexes in a nuclear extract, avoiding ion-exchange chromatography and high salt concentration to avoid dissociation of subunits (18). Under these conditions they found that the majority of each Mediator subunit, including Srb8 to Srb11, was associated with Pol II in a complex of ~1.9 MDa that lacks GTFs. A less abundant complex of ~0.55 MDa included a subset of Mediator subunits.

Several mammalian multiprotein complexes have been identified that have several subunits homologous to components of the yeast Mediator and several subunits that are not clearly related to yeast proteins (21). Broadly speaking, two size classes of complexes have been identified. Complexes of ~2 MDa, such as the TRAP/SMCC (12), NAT (32), DRIP (30), ARC (25), and human Mediator (2) complexes, share an overlapping set of components. Smaller complexes (~500 to 700 kDa) containing Srb/Med homologs have also been identified, including the murine Mediator (13), CRSP (31), and PC2 (20) complexes. These mammalian Mediator-like complexes were identified and purified by different biochemical procedures. TRAP (7), DRIP (30), ARC (25), and human Mediator (2) were purified on the basis of their ability to bind to activation domains during affinity chromatography. SMCC (8) and NAT (32) were purified based on their content of CDK8, which is a homolog of yeast Srb10. Functions of these Mediator complexes were assayed in different in vitro transcription systems that varied in the purity of the GTFs and the use of naked DNA versus chromatin templates. Most of these complexes, including ARC, DRIP, PC2, and CRSP, greatly stimulated activated transcription (21). NAT and SMCC repressed activated transcription in assays with highly purified factors (8, 32), but SMCC activated transcription when TFIIH was omitted (8). This repression has been attributed to the phosphorylation of the cyclin H subunit of TFIIH by the CDK8 kinase within

* Corresponding author. Mailing address: University of California Los Angeles, Molecular Biology Institute, 611 Charles Young Dr. E, Box 951570, Los Angeles, CA 90095-1570. Phone: (310) 206-6298. Fax: (310) 206-7286. E-mail: berk@mbi.ucla.edu.

the Mediator complexes (1). The human Mediator complex inhibited activated transcription in a highly purified system but stimulated high levels of activated transcription in reactions with partially purified GTFs (2).

While the different mammalian Mediator-like complexes so far described share many subunits, they also differ with regard to their reported subunit composition (21). This raises the question whether there are multiple distinct Mediator-like complexes in mammalian cells that may differ in their functional properties or whether there is in fact one or a small number of mammalian Mediator complexes. In the latter case, the apparent differences in subunit composition reported by different laboratories might result from relatively minor differences in the methods used to characterize the subunits or from different methods of purification that partially dissociate a single large complex. To estimate the number of different complexes containing Mediator subunits in HeLa cells, we subjected unfractionated HeLa cell nuclear extract to gel filtration chromatography at low salt concentration to avoid the dissociation of subunits. Protein complexes in eluted fractions were characterized by immunoblotting with several antibodies specific for Mediator subunits, including components of both size classes of Mediator complexes described. A single peak of ~2 MDa containing each of the several Mediator subunits was observed. No significant peak was observed at ~500 to 700 kDa, indicating that either this size class of Mediator complex is much less abundant than the ~2-MDa size class or that the ~500 to 700-kDa Mediator complexes described above were derived from the larger size class by dissociation during the multiple steps of column chromatography used in their purification. Mediator subunits CDK8, cyclin C, and hSur2 were also observed in lower-molecular-mass complexes, but only the ~2-MDa size class significantly stimulated activated transcription. High-resolution gel filtration and immunoprecipitation analyses indicated that there are at least two subclasses of ~2-MDa Mediator complexes, one containing CDK8 and cyclin C and one lacking these subunits. A total of ~300,000 hSur2 subunits per cell were present in the ~2-MDa Mediator complexes; this number is approximately equal to the number of Pol II molecules per HeLa cell (15).

MATERIALS AND METHODS

Nuclear extract preparation and chromatography on Superose 6. HeLa cell nuclear extract was prepared as described previously (6), except that the final dialysis in 0.1 M KCl D buffer (20 mM HEPES) [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol) was omitted. A 2-ml volume of undialyzed nuclear extract was directly loaded onto a 100-ml Superose 6 column (HR 16/50; Pharmacia) preequilibrated with 0.3 M KCl D buffer and run in the same buffer. Column fractions of 1 ml were collected.

Immunoblotting. Superose 6 column fractions (100 μl) were precipitated with trichloroacetic acid, and the precipitate was dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved on a 10% polyacrylamide gel. The gels were electroblotted onto a nitrocellulose membrane, and the membranes were blocked by incubation at room temperature (RT) for 1 h in TBS (150 mM NaCl, 10 mM Tris [pH 7.4]) containing 5% nonfat dry milk. Rabbit polyclonal antibodies against CDK8 and cyclin C, DRIP 150, and hMed 6 were kindly provided by Emma Lees, Leonard Freedman, and Danny Reinberg, respectively. Antibodies to P300 and HDAC2 were from Santa Cruz Biotechnology. Monoclonal antibody to Pol II large subunit (8WG16) was from Thompson et al. (35). Monoclonal antibody to hSur2 was developed in collaboration with Liangru Shi, BD Pharmingen. Immunoblots were developed using enhanced chemiluminescence reagents from Pierce. Other protein fractions were diluted with an equal volume of 2× SDS-PAGE sample buffer and analyzed similarly.

Recombinant hSur2 baculovirus production and purification. An hSur2-expressing baculovirus was generated with the Bac to Bac baculovirus production system (Gibco-BRL) as specified by the manufacturer. Briefly, hSur2 cDNA (2) was cloned into the pBacHta plasmid to generate an N-terminal 6His-tagged fusion. This plasmid was introduced into DH5-Bac cells to produce the 6His-hSur2-baculovirus plasmid (prSur2-BAC). prSur2-BAC was transfected into Sf9 cells to recover recombinant baculovirus Sur2-BAC. Then 2×10^8 Sf9 cells were infected with Sur2-BAC at a multiplicity of infection of 2, harvested 48 h post-infection, and lysed in 3 ml of 6 M guanidine HCl–0.1 M sodium phosphate–0.01 M Tris-Cl (pH 8.0) for 30 min at RT. After centrifugation at 10,000 × *g* for 30 min, the supernatant was bound to 2 ml of Ni^{2+} resin (Qiagen) in batch at RT for 1 h. The resin was washed three times in 8 M urea–0.1 M sodium phosphate–0.01 M Tris-Cl–0.01 M imidazole (pH 8.0). rSur2 was then eluted from the resin in 2 ml of SDS-PAGE sample buffer and incubated at 100°C for 5 min. To estimate the concentration of r-hSur2, 20, 30, and 40 μl were subjected to SDS-PAGE (10% polyacrylamide) with 50 to 500 ng of bovine serum albumin in 50-ng increments. The gel was stained with Coomasie blue, revealing that 20 μl of r-hSur2 produced a stained band of equal intensity to 100 ng of bovine serum albumin, corresponding to a concentration of 5 ng/μl.

Immunoprecipitation. Pooled ~2-MDa or ~150-kDa fractions (400 to 800 μl) from the Superose 6 column were made 1% in NP-40 and immunoprecipitated with 20 μl of agarose-conjugated goat anti-CDK8 antibody or goat normal immunoglobulin G (IgG) (Santa Cruz Biotechnology). The pelleted agarose beads were washed three times with 0.3 M KCl D buffer plus 1% NP-40 and once with phosphate-buffered saline before elution in SDS-PAGE sample buffer and immunoblotting. Immunodepletion of CDK8 from the ~2-MDa fraction was performed by repeating the immunoprecipitation five times with 20 μl of goat anti-CDK8 or normal goat IgG beads. The final supernatants from the last immunoprecipitation were trichloroacetic acid precipitated and subjected to SDS-PAGE and immunoblotting.

Concentration of Superose 6 column fractions. Fractions containing ~2-MDa Mediator complexes identified by immunoblotting from three runs of the Superose 6 column (e.g., fractions 41 to 50 in Fig. 1A) were pooled, dialyzed into 0.1 M KCl D buffer, and then bound to a 300-μl Whatman P11 phosphocellulose column equilibrated in 0.1 M KCl D buffer. After the column was washed with 1 ml 0.1 M KCl D buffer, Mediator complex was eluted with 0.5 M KCl D buffer and fractions of 1 drop (~50 μl) were collected. The protein peak (~150 μl) was dialyzed into 0.1 M KCl D buffer. Fractions containing hSur2, CDK8, and cyclin C eluting at ~150 kDa from three Superose 6 column runs (e.g., Fig. 1A fractions 65 to 73) were pooled, and half of the pool was concentrated 25-fold to 150 μl using a Microcon 10 centrifugal filter device (Amicon).

Phosphocellulose chromatography of the ~150-kDa Superose 6 fraction. The other half of pooled ~150-kDa Superose 6 column fractions was dialyzed into 0.1 M KCl D buffer and applied to a 300-μl P11 column in 0.1 M KCl D buffer. The flowthrough contained hSur2 and was concentrated 25-fold to 150 μl by centrifugation through a Microcon 10 device as above. The bound fraction was eluted with 0.5 M KCl D buffer, and the 150-μl protein peak was dialyzed into 0.1 M KCl D buffer.

In vitro transcription. Recombinant TFIIB was purified as described previously (22). Gal4-VP16 (amino acids 1 to 147 of Gal4 fused to amino acids 413 to 490 of VP16) was expressed in *Escherichia coli* and purified as described previously (33). GAL4-E1A (amino acids 1 to 147 of Gal4 fused to amino acids 121 to 223 of the adenovirus 2 large E1A protein) was expressed in *E. coli* and purified as described previously (42) except that SP-Sepharose (Pharmacia) was used and washed with 0.2 M KCl D buffer and eluted with 0.5 M KCl D buffer. Protein fractions AB, DB, and CBS and hMediator fraction Q were purified from HeLa nuclear extract as described previously (2), except that the ~2-MDa hMediator was concentrated on phosphocellulose rather than HiTrap Q. In vitro transcription reaction mixtures contained rTFIIB (45 ng), protein fractions AB (1.8 μg), DB (2.1 μg), and CBS (5.2 μg) in 35 μl of 33 mM HEPES (pH 7.9), 60 mM KCl, 0.12 mM EDTA, 12% glycerol, 8 mM $MgCl_2$, 15 μM $ZnCl_2$, 4% polyethylene glycol 8000, 45 mM β-mercaptoethanol, 30 U of RNasin (Promega), 100 μM each ATP and UTP, 3 μM CTP, 50 μM 3′-*O*-methyl-GTP, 10 μCi, of [α-^{32}P] CTP (3,000 Ci/mmol; NEN), 60 ng of pG5ΔMLP, and 60 ng of pΔMLP. Where indicated, the Mediator Q fraction (7.5 μg), Gal4-VP16 (40 ng), Gal4-E1A (36 ng), or the indicated amounts of protein fractions were added. The reaction mixtures were incubated at 30°C for 1 h and treated with 20 U of RNase T_1 (Boehringer Mannheim) at 37°C for 5 min, and the reactions were stopped by the addition of an equal volume of 1% SDS–200 mM NaCl–20 mM EDTA–proteinase K (10 μg/ml; Boehringer Mannheim); the mixtures were then incubated at 37°C for 10 min. Transcripts were extracted with phenol-chloroform-

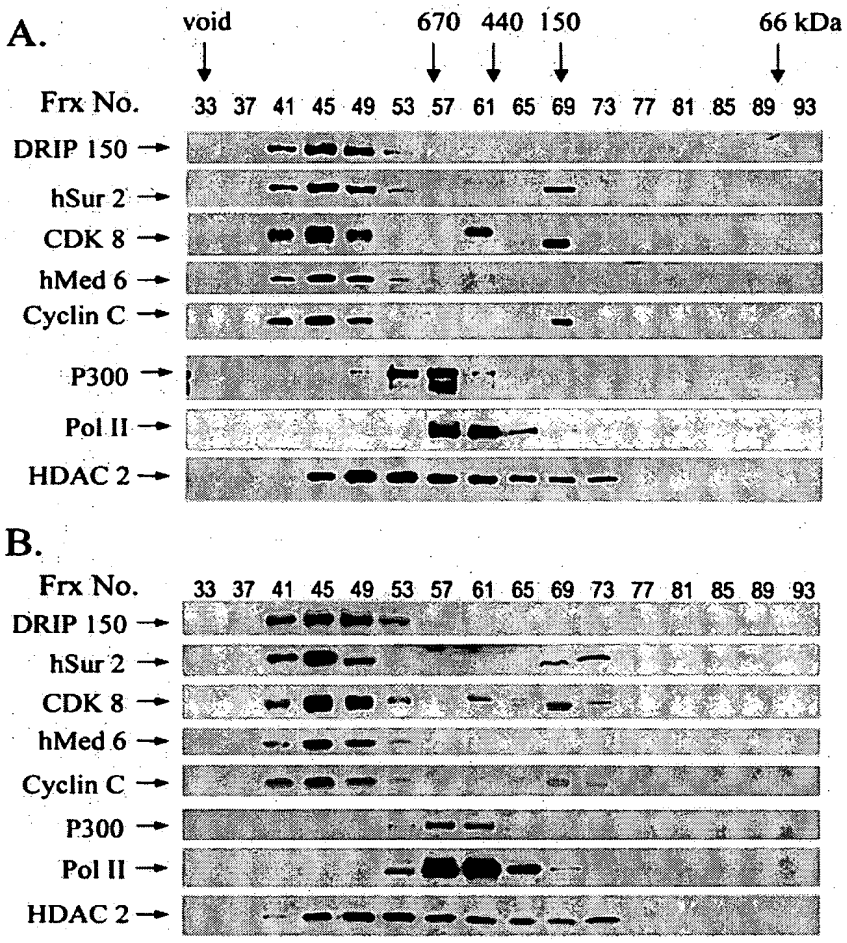

FIG. 1. Gel filtration chromatography of Mediator subunits in HeLa nuclear extract. A Superose 6 column was run in 0.3 M KCl (A) or 1.0 M KCl (B) in buffer D. Every fourth column fraction was analyzed by SDS-PAGE and immunoblotting with antibodies against DRIP150, hSur2, CDK8, cyclin C, hMed 6, P300, RPB1 of Pol II, or HDAC2, as indicated. Column fractions in which the peaks of protein standards (670, 440, 150, and 66 kDa) eluted and the void volume are indicated.

isoamyl alcohol, ethanol precipitated, and run on an 8% polyacrylamide–8 M urea–TBE gel. The gels were dried and exposed to film with an intensifying screen.

RESULTS

Fractionation of HeLa cell nuclear extract by gel filtration. As an initial approach to determining whether single or multiple types of complexes containing Mediator subunits exist in HeLa cells, nuclear extract with high activity for specific initiation by Pol II was prepared as described by Dignam et al. (6). The extracted proteins were directly fractionated by size on a gel filtration column run in a low-salt buffer, and Mediator components in column fractions were detected by immunoblotting. This procedure avoids ion-exchange chromatography and exposure to high salt concentrations that might dissociate subunits from a large, multiprotein complex. In the Dignam et al. procedure, isolated nuclei are extracted in a buffer containing 0.3 M NaCl (6). This extract was directly applied to a Superose 6 column without dialysis because we found that dialysis into a buffer containing 0.1 M KCl or dilution to 0.1 M NaCl invariably resulted in a precipitate that contained a large percentage of the Mediator components in the initial, undialyzed nuclear extract. The Superose 6 column was run in a buffer containing 0.3 M KCl, a buffer of comparable ionic strength to the nuclear extract and compatible with those used in assays of in vitro transcriptional activity. We analyzed subunits that are components of both the large (~1 to 2-MDa) complexes and the smaller (~500 to 700-kDa) complexes described recently (DRIP150 and hSur2), as well as hMed6, a component of all the large complexes recently described, but only some of the smaller complexes and CDK8 and cyclin C, described as components of some of the large Mediator complexes but not others (21).

A major peak at ~2 MDa was observed for all of the Mediator components analyzed (Fig. 1A). A second, smaller peak at ~150 kDa was also detected that contained hSur2, CDK8, and cyclin C. CDK8 was also detected in a species that eluted at ~500 kDa. The CDK8 proteins observed in species of ~2

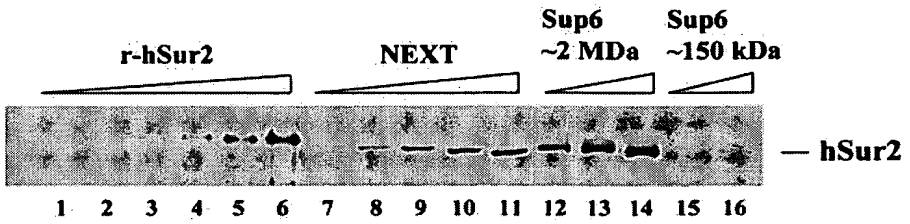

FIG. 2. Quantitation of hSur2 in nuclear extract and Superose 6 column fractions. Immunoblotting was performed with r-hSur2 (lanes 1 to 6, containing 17, 33, 67, 100, 133, and 167 fmol of r-hSur2, respectively), nuclear extract (lanes 7 to 11, containing 1, 2, 4, 6, and 8 μl, respectively), pooled Superose 6 fractions 41 to 50 (Fig. 1A) (lanes 12 to 14, containing 50, 100, and 200 μl, respectively), and pooled Superose 6 fractions 65 to 74 (lanes 15 and 16, containing 50 and 100 μl, respectively). A 1-μl volume of nuclear extract was derived from 1.3×10^4 cells; a 1-μl volume of Superose 6 fraction pools was derived from 3.9×10^3 cells.

MDa, ~500 kDa, and ~150 kDa had slightly different mobility in the SDS gel, suggesting that they differed in posttranslational modifications such as phosphorylation. Each of these immunoblot signals probably represents CDK8 rather than a cross-reacting protein, since they were each detected with separate anti-CDK8 antibodies prepared in rabbits and goats (data not shown). Other high-molecular-mass multiprotein complexes (Pol II and complexes containing P300 and histone deacetylase 2 [HDAC2] clearly fractionated differently from the ~2-MDa Mediator complexes, demonstrating the resolution of the column in this size range. HDAC2 is an example of a protein known to be associated with at least two different multisubunit complexes, the Sin3A-HDAC complex (41) and the NURD complex (36, 39, 40). It was detected in multiple column fractions ranging in size from 140 kDa to nearly 2 MDa. It is unlikely that the apparent molecular mass of the ~2-MDa Mediator complex was influenced by binding to DNA in the initial extract because it eluted from the column at exactly the same position as highly purified hMediator (Q fraction), whose purification includes anion-exchange columns that remove high-molecular-mass nucleic acids (2).

A peak of DRIP150 and hSur2, both components of the PC2 and CRSP complexes (21), was not apparent in fractions eluting at ~500 to 700 kDa (Fig. 1A), the size of the PC2 and CRSP complexes determined by gel filtration (20, 31), even when every fraction was analyzed. Prolonged exposures of the immunoblots revealed the trailing edge of the ~2-MDa complex peak extending into fractions from the region of the column corresponding to ~500 kDa. However, a peak corresponding to a protein complex of ~500 to 700 kDa was not apparent even after prolonged exposures. We estimate that we would have been able to detect a complex of ~500 kDa if it were present at 1/10 the level of the major ~2-MDa complex or more.

The Superose 6 column analyzed in Fig. 1A was run in a buffer of 0.3 M KCl to avoid possible dissociation of subunits that might occur at higher salt concentrations. However, we found that the Mediator subunit-containing multiprotein complexes were stable even at high salt concentration since a virtually identical elution profile was observed when gel filtration was performed with a buffer containing 1 M KCl (Fig. 1B). At both 0.3 and 1.0 M KCl, the vast majority of Pol II in the nuclear extract fractionated at ~600 kDa, the size of the highly purified, 12-subunit "core" Pol II. P300 apparently dissociated from an interacting protein(s) in 1 M KCl since it eluted at a lower apparent molecular mass when the column was run in 1 M KCl (~600 kDa) than when it was run in 0.3 M KCl (~800 kDa). It seemed possible that the failure to detect the Mediator-like complexes of ~500 to 700 kDa in the column run in 0.3 M KCl could have been because they bound to high-molecular-mass DNA in the Dignam et al. extract. However, the failure to detect this size complex in the column run in 1 M KCl argues against this possibility since most protein-DNA interactions are disrupted at this high salt concentration.

Number of Mediator complexes in a HeLa cell. It is of interest to estimate the approximate number of Mediator complexes per HeLa cell. If there were fewer Mediator complexes than the number of transcribed genes, it would be unlikely that Mediator complexes are generally required for regulated transcription. To make this estimate, we counted the number of hSur2 molecules in a single HeLa cell by quantitative immunoblotting using as a standard hSur2 expressed from a baculovirus vector and purified using an appended 6His tag. The concentration of purified r-hSur2 was estimated by comparing the intensities of a dilution series with a titration of known amounts of bovine serum albumin on a Coomassie blue-stained SDS gel. Immunoblotting was then performed using a dilution series of the r-hSur2, undialyzed HeLa cell nuclear extract, and pooled Superose 6 fractions containing hSur2. Based on the relative intensities of bands and the number of cells from which the nuclear extract and Superose 6 fractions were derived (Fig. 2), we estimate that there are 300,000 to 500,000 molecules of hSur2 per HeLa cell. A whole-cell extract was also prepared by lysing cells directly in SDS sample buffer, yielding an estimate of 600,000 molecules of hSur2 per HeLa cell (data not shown). Consequently, most of the hSur2 and therefore most of the Mediator is efficiently extracted from nuclei by the Dignam et al. procedure (6). Of the total hSur2 molecules, ~300,000 were recovered in the ~2-MDa Mediator fraction in repeated analyses, whereas fewer than 100,000 copies of hSur2 were recovered in the ~150-kDa Superose 6 fractions. In several independent analyses, the number of hSur2 molecules in the ~2-MDa Mediator complexes was four to six times in excess of the number of hSur2 molecules in the ~150-kDa species.

Analysis of the ~150-kDa fraction. When nuclear extract was directly fractionated on Superose 6 in 0.3 M KCl, a small fraction of the hSur2, CDK8, and cyclin C coeluted in a fraction corresponding to ~150 kDa (Fig. 1A). To determine if the hSur2 in this fraction was in a complex containing CDK8 and

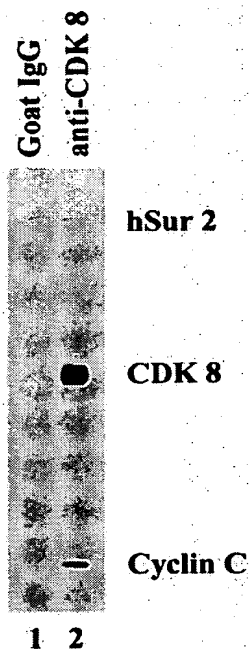

FIG. 3. Immunoprecipitation of the 150-kDa Superose 6 column fractions. Superose 6 column fractions 65 to 74 (Fig. 1A) were pooled and subjected to immunoprecipitation with control normal goat IgG or goat anti-CDK8 IgG. The immunoprecipitates were resolved on a 10% SDS gel and subjected to immunoblotting with anti-hSur2, anti-CDK8, or anti-cyclin C as indicated.

cyclin C, proteins in the fraction were subjected to immunoprecipitation using anti-CDK8 antibody. The anti-CDK8 antibody coprecipitated the CDK8-cyclin C pair, but hSur2 did not precipitate above a low background level observed with control antibody (Fig. 3). Additionally, when the 150-kDa Superose 6 fraction was dialyzed into a buffer containing 0.1 M KCl and fractionated over a phosphocellulose column, hSur2 was found in the flowthrough whereas CDK8 and cyclin C bound to the column and were step eluted with 0.5 M KCl D buffer (Fig. 4). Taken together, these results indicate that CDK8 and cyclin C are associated with each other in the ~150-kDa Superose 6 fraction but that hSur2 in this fraction is not in the same protein complex. Rather, hSur2 fortuitously cofractionates with the CDK8-cyclin C-containing complex on Superose 6 in 0.3 M KCl. Consistent with this, when the nuclear extract was chromatographed on Superose 6 in 1 M KCl, the CDK8-cyclin C-containing complex peaked in fraction 69 whereas hSur2 peaked in fraction 73 (Fig. 1B). Since the molecular mass of hSur2 predicted by the amino acid sequence derived from a full-length cDNA clone (2) is ~150 kDa, it seems likely that hSur2 in the 150-kDa Superose 6 fraction is monomeric hSur2 unassociated with other polypeptides. This is probably the source of monomeric hSur2 that binds to an E1A activation domain affinity column (2). The sum of the molecular masses of CDK8 and cyclin C is less than 90 kDa. Therefore it seems likely that one or more additional polypeptides associate with CDK8 and cyclin C in the Superose 6 150-kDa fraction.

Heterogeneity in the ~2-MDa Mediator complexes. When multiple small fractions across the peak of the ~2-MDa Mediator complex were analyzed by immunoblotting for Mediator subunits, the peak of CDK8 and cyclin C was reproducibly observed centered about two fractions earlier in the elution profile than the peaks of DRIP150, hSur2, and hMed6, which precisely coeluted (Fig. 5). This result suggested to us that the high-molecular-mass Mediator complexes might be heterogeneous, with complexes containing CDK8 and cyclin C eluting in an overlapping peak just ahead of complexes lacking the CDK8-cyclin pair. To test this possibility, the ~2-MDa Mediator-containing Superose 6 fractions were pooled and immunoprecipitated with anti-CDK8 antibody. As expected, multiple Mediator subunits coimmunoprecipitated with CDK8 but not with control goat IgG (Fig. 6A). However, after multiple rounds of immunodepletion with the anti-CDK8 antibody, multiple Mediator components remained in the supernatant while CDK8 and cyclin C were substantially decreased. Naar et al. (25) also reported that CDK8 was substoichiometric compared to other subunits of the ARC complex and could be immunodepleted without depleting the other subunits. Based on these findings, we conclude that Mediator complexes in HeLa nuclear extract are heterogeneous and can be classified into at least two types: one containing CDK8-cyclin C that can be immunoprecipitated with anti-CDK8 antibody, and one lacking CDK8 and cyclin C that remains in the supernatant after immunodepletion with anti-CDK8 antibody.

TRAP, SMCC, NAT, and human Mediator complexes were each reported to contain CDK8 and cyclin C (2, 8, 12, 32), while a large fraction of the ARC complex was reported to lack CDK8 (25). The DRIP and ARC complexes were purified by affinity chromatography on activation domains of the vitamin D_3 receptor and SREBP-1a, respectively, and are reported to be identical or nearly so (25, 30). We wondered whether these activation domains might selectively bind the class of Mediator

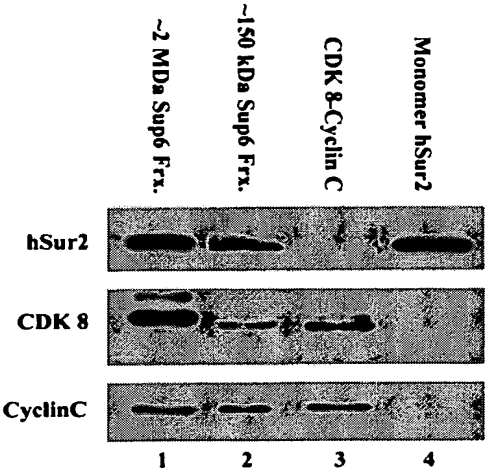

FIG. 4. Immunoblotting of concentrated Superose 6 fractions and 150-kDa phosphocellulose fractions. A 10-μl volume each of the pooled and concentrated ~2-MDa Superose 6 fractions (lane 1), ~150 kDa Superose 6 fractions (lane 2), the phosphocellulose-bound fraction from the pooled ~150-kDa Superose 6 fractions (lane 3), and the concentrated phosphocellulose flowthrough from the ~150-kDa Superose 6 fractions (lane 4) was resolved by SDS-PAGE (10% polyacrylamide) and subjected to immunoblotting with antibody to hSur2, CDK8, or cyclin C, as indicated.

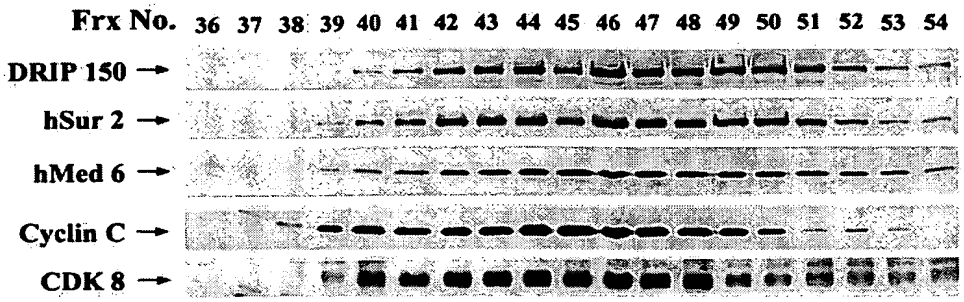

FIG. 5. High-resolution analysis of the ~2 MDa Superose 6 fraction. HeLa nuclear extract was chromatographed on Superose 6 as in Fig. 1A. Then 100 μl of each 1-ml fraction was subjected to immunoblotting using the indicated antibodies.

complexes described above that lacks CDK8 and cyclin C. To test this possibility, Mediator complexes were purified directly from nuclear extract by binding to a VDR ligand binding domain (LBD) affinity column in the presence of vitamin D_3, followed by elution with a peptide corresponding to the high-affinity LXXLL-motif LBD-binding region of DRIP205, as described previously (29). The resulting, extensively purified protein fraction had high activity for stimulating in vitro transcription activated by Gal4-VP16 (data not shown). Immunoblotting revealed that CDK8 and cyclin C copurified with hSur2 on the VDR LBD affinity column, just as they did during purification by conventional chromatography (Fig. 6B) and on affinity columns of the E1A and VP16 activation domains (2). Consequently, it does not appear that the VDR LBD selectively binds the class of mammalian Mediator complexes lacking CDK8 and cyclin C.

Transcriptional activity of Mediator subunit-containing fractions. The Mediator subunit-containing Superose 6 fractions of ~2 MDa and ~150 kDa were concentrated and analyzed for their influence on activated transcription in vitro. In vitro transcription reactions used partially purified Pol II and GTFs fractionated on phosphocellulose and DEAE-Sepharose, and separated from the ~2 MDa Mediator complexes by gel filtration, as described previously (2). Equimolar amounts of two templates were included in the transcription reaction mixtures. One (ΔMLP) contained only the adenovirus type 2 major late promoter (MLP) to assay basal transcription. Transcription from this template generated a 400-nucleotide G-less transcript. The other (G_5ΔMLP) contained the MLP with five upstream Gal4-binding sites to assay transcription activated by Gal4–DNA-binding domain fusion proteins and generated a 200-nucleotide G-less transcript.

As reported earlier, a highly purified human Mediator fraction (Q-fraction [2]) greatly stimulated transcription activated by Gal4-VP16 in this system (Fig. 7, lane 3). When the nuclear extract-derived Superose 6 ~2-MDa fraction (Fig. 1A) containing the same amount of hSur2 as determined by immunoblotting (data not shown) was added to the reaction mixture, comparable stimulation of activated transcription was observed (lane 4). In contrast, addition of a comparable amount of hSur2 in the ~150 kDa Superose 6 fraction (Fig. 4, lanes 1 and 2) inhibited both basal and activated transcription (Fig. 7, lane 6). As shown above, apparently monomeric hSur2 and a complex containing CDK8 and cyclin C in the ~150-kDa Superose 6 fraction could be separated by chromatography on phosphocellulose. Addition of the phosphocellulose fraction containing monomeric hSur2 (Fig. 4, lane 4) had a very modest stimulatory affect on activated in vitro transcription (Fig. 7, lane 5), whereas the phosphocellulose fraction containing CDK8 and cyclin C derived from the ~150-kDa Superose 6 fraction (Fig. 4, lane 3) inhibited both basal and activated transcription (Fig. 7, lane 7). However, components of this fraction in addition to CDK8-cyclin C were responsible for this inhibition, since a similar extent of inhibition was observed when CDK8 and cyclin C were depleted by immunoprecipitation with anti-

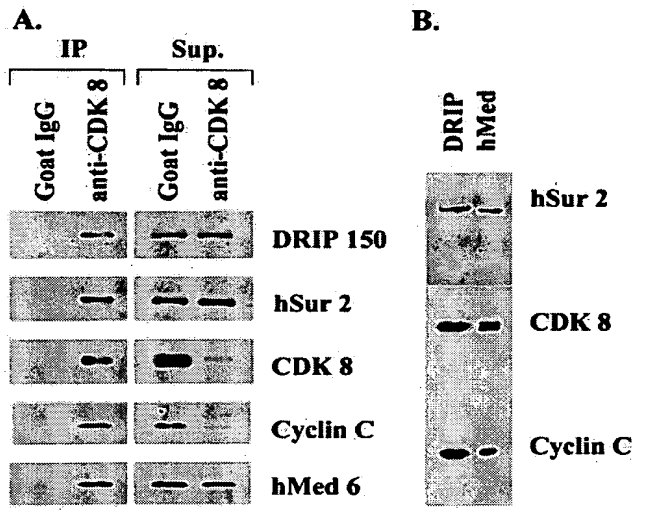

FIG. 6. Immunoblots of anti-CDK8 immunoprecipitation pellet, supernatant, and DRIP complex. (A) Immunodepletion of ~2-MDa Mediator complexes with anti-CDK8 antibody. Fractions 40 to 50 from the Superose 6 column shown in Fig. 5 were pooled and subjected to immunoprecipitation with anti-CDK8 antibody or control normal goat IgG. The immunoprecipitate (IP) from the first immunoprecipitation and the supernatant (Sup.) following the fifth immunoprecipitation were resolved by SDS-PAGE and subjected to immunoblotting with the indicated antibodies. (B) Mediator complexes containing CDK8 and cyclin C are bound by the VDR LBD. Nuclear extract was incubated with GST-VDR(LBD) bound to glutathione-coupled agarose beads. The beads were washed extensively and eluted with 100 μM NR2 peptide corresponding to the high-affinity binding site in DRIP150, as described previously (29). The eluate (DRIP) and purified human Mediator (Q-fraction [2]) were subjected to SDS-PAGE and immunoblotting with the indicated antibodies.

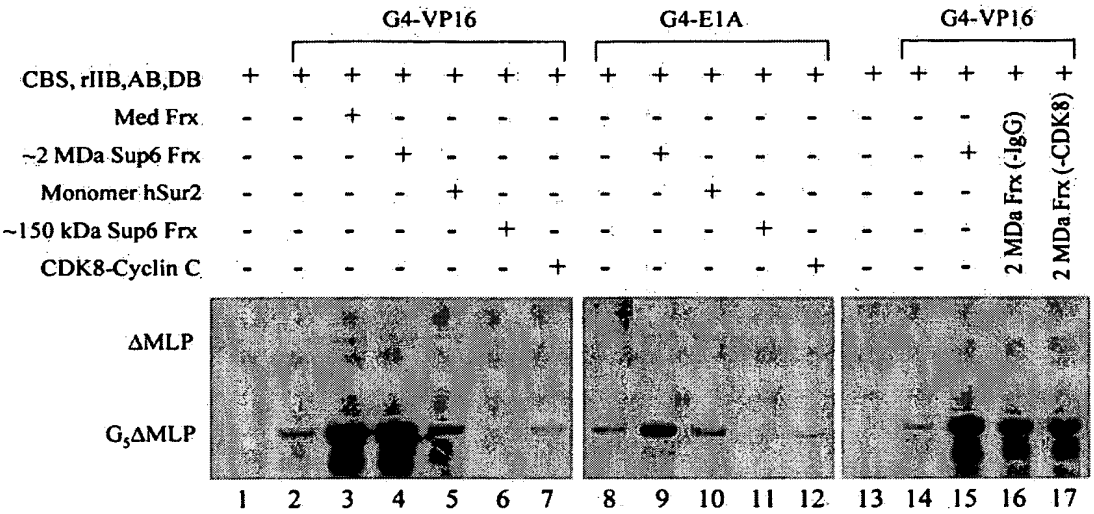

FIG. 7. Transcriptional activities of protein fractions. ΔMLP indicates the position of the 400-nucleotide G-less transcript transcribed from the template lacking Gal4-binding sites. G_5ΔMLP indicates the position of the 200-nucleotide G-less transcript from the template containing five Gal4-binding sites. Transcription reaction mixtures contained 8 μl of the indicated protein fractions analyzed by immunoblotting in the experiment in Fig. 4. Reaction mixtures in lanes 2 to 7 contained Gal4-VP16, and reaction mixtures in lanes 8 to 12 contained Gal4-E1A. Med Frx (lane 3) refers to the Q-fraction of purified Mediator equivalent to the same amount of ~2-MDa Mediator as used in lanes 4 and 9, as determined by immunoblotting. Reaction mixtures in lanes 13 to 17 were from a separate experiment with the ~2-MDa Superose 6 fraction (lane 15) and the same fraction immunodepleted with control goat IgG (lane 16) or goat anti-CDK8 antibody (lane 17).

CDK8 antibody (data not shown). Similar results were observed for in vitro transcription reaction mixtures containing Gal4-E1A (Fig. 7A, lanes 9 to 12). The ~2-MDa Superose 6 fraction depleted of CDK8-cyclin C-containing complexes (Fig. 6A, lane 4) had similar activity to the control fraction subjected to immunodepletion with control IgG (Fig. 6A, lane 3; Fig. 7, lanes 16 and 17). The slight decrease in activity compared to the untreated fraction (Fig. 7, lane 15) was due to dilution of the fractions by the immunoprecipitation procedure. Similar results were reported by Naar et al. (25) for the ARC complex immunodepleted of CDK8.

In summary, the partially purified ~2-MDa Mediator complexes derived directly from nuclear extract had transcription-stimulating activity comparable to that of highly purified human Mediator whereas the apparently monomeric hSur2 had relatively little effect. The ~150-kDa complex containing CDK8 and cyclin C inhibited both basal and activated transcription.

DISCUSSION

The principal result of this study is that the major mammalian Mediator complexes observed in HeLa cell nuclear extract are high-molecular-mass (~2-MDa) complexes as determined by gel filtration (Fig. 1). There have been several recent reports of multiprotein complexes isolated from mammalian cells that contain homologs of *S. cerevisiae* Mediator subunits. Several of these are functionally related to yeast Mediator in that they greatly stimulate activated transcription in vitro. These complexes fall into two groups on the basis of their size and complexity (21). The members of one group, including TRAP/SMCC (12), NAT (32), ARC (25), DRIP (30), and Mediator (2), were estimated to be ~1 to 2 MDa by gel filtration and were reported to contain 12 to 30 polypeptides. A second, smaller size class included murine Mediator (13), CRSP (31), and PC2 (20), and these were reported to have 8 to 12 subunits, including a subset of those found in the larger Mediator complexes. CRSP and PC2 were reported to be ~600 to 700 and ~500 kDa, respectively, as determined by gel filtration. Complexes of ~500 to 700 kDa containing Mediator subunits reported to be in the highly purified CRSP and PC2 complexes (DRIP150 and hSur2) (21) were not detected in the Superose 6 profile of HeLa nuclear extract (Fig. 1). The distribution of Mediator subunits in column fractions was clearly different from that of HDAC2 (Fig. 1), an example of a protein known to be a component of at least two different multisubunit complexes (36, 39–41). Consequently, the ~500-kDa mammalian Mediator complexes are much less abundant in nuclear extract than are the ~2 MDa complexes. Since hSur2, a mammalian Mediator subunit reported to be in both size classes of isolated complexes, was efficiently extracted during nuclear extract preparation, the ~500-kDa size class of Mediator complexes are likely to be much less abundant than the ~2-MDa size class in vivo as well.

The high-molecular-mass mammalian Mediator complexes recently described have many subunits in common. However, their reported subunit compositions are not identical, as summarized by Malik and Roeder (21). Is this because several distinct ~2-MDa complexes that share some subunits but differ in others occur in HeLa cells and were purified separately by different groups? Or do the differences in composition reported in these initial studies result from the technical challenges of fully characterizing these low-abundance, multisubunit complexes?

To search for heterogeneity in Mediator complexes in the ~2-MDa size range, we collected multiple small fractions across the Superose 6 ~2-MDa peak and analyzed the elution

profiles of several Mediator subunits. The simplest interpretation of our data is that there are two types of ~2-MDa Mediator complexes. The peaks of DRIP150, hSur2, and hMed6 were similar in shape to the peaks of homogeneous molecular mass marker proteins analyzed on the same column (data not shown). On the other hand, CDK8 and cyclin C were shifted to an overlapping position in the profile centered at a slightly higher molecular mass than the coeluting peaks of DRIP150, hSur2, and hMed6 (Fig. 5). This suggested that CDK8 and cyclin C might be associated with a subclass of Mediator complexes, increasing the molecular mass of these complexes and causing them to elute from the column slightly earlier. Consistent with this possibility, depletion of CDK8 from the Superose 6 ~2-MDa fraction by repeated immunoprecipitation with anti-CDK8 antibody extensively depleted CDK8 and cyclin C but not DRIP150, hSur2, or hMed6. Similarly, Naar et al. (25) reported that depletion of a substoichiometric amount of CDK8 from purified ARC complex did not deplete other ARC subunits, nor did it alter the transcription-stimulating activity of the complex. We also found that depletion of the CDK8-cyclin C-containing complexes by immunoprecipitation did not significantly alter the transcription-stimulating activity of the ~2-MDa fraction containing the human Mediator complex (Fig. 7).

All the Mediator subunits analyzed were specifically immunoprecipitated by anti-CDK8 antibody, consistent with the model that one class of Mediator complex contains CDK8 and cyclin C while a second class does not. We think that an alternative model is unlikely, i.e., that all Mediator complexes contain weakly bound CDK8-cyclin C that is stripped from a large fraction of the complexes during immunoprecipitation with anti-CDK8 antibody. As discussed above, the gel filtration data are more consistent with two types of complexes, one containing and one lacking CDK8-cyclin C. Based on the separation of the peak fractions of cyclin C and hSur2, the apparent difference in molecular mass of complexes containing and lacking CDK8-cyclin C is ~200 kDa, larger than the sum of the molecular masses of CDK8 and cyclin C. This suggests that the larger complexes may include additional subunits that are missing from the smaller complex besides CDK8 and cyclin C. The yeast Mediator complex lacking Srb10 and Srb11, the homologs of CDK8 and cyclin C, also lacks Srb8 and Srb9 found in other Mediator-containing complexes (23). Srb8 to Srb11 appear to form a subcomplex of Pol II holoenzyme required for glucose repression (3, 18, 23). Moreover, Holstege et al. (11) reported that the Srb10 concentration falls as yeast cells deplete nutrients from their media whereas the concentrations of Mediator subunits other than the Srb8 to Srb11 module remain constant. Consequently, it appears that the Srb8 to Srb11 module can be expressed or not depending on cellular physiology. By analogy to yeast Mediator, the proportion of HeLa Mediator complex that contains or lacks a CDK8-cyclin C module may depend on the growth conditions and may vary among strains of HeLa cells.

While the model of two types of human Mediator complexes is the simplest interpretation of our data, gel filtration is not capable of clearly distinguishing megadalton complexes that differ from each other by a few hundred kilodaltons. Consequently, our data are not inconsistent with the existence of multiple types of mammalian Mediator complexes. Nonetheless, it remains quite possible that the apparent differences between the Mediator complexes described in recent reports are due to the technical difficulties of fully characterizing all of the low-abundance subunits. Further data, such as the immunodepletion study reported here using anti-CDK8 antibody, are necessary for a demonstration that there is additional heterogeneity between mammalian Mediator complexes.

Superose 6 fractions of nuclear extract with a CDK8-cyclin C-containing complex of ~150 kDa inhibited basal and activated transcription (Fig. 7). This is not surprising since it has been shown recently that CDK8 phosphorylates the cyclin H subunit of TFIIH, inhibiting its function in transcription (1). Similarly, ~2-MDa mammalian Mediator complexes containing CDK8 and cyclin C inhibit transcription in reactions with highly purified Pol II and GTFs (2, 8, 32), and this inhibition depends on the protein kinase activity of CDK8 (1). However, in reactions with partially purified GTFs, Mediator complex containing CDK8 and cyclin C greatly stimulates activated transcription (2). These results imply that a factor(s) in the partially purified GTFs controls CDK8 activity so that it does not inhibit TFIIH function in the absence of an appropriate signal from a repressor. The ability of CDK8 to inhibit transcription in the purified system may explain the purification of the PC2 complex (20). Our observation that the concentration of ~500-kDa Mediator subunit-containing complexes in nuclear extract is very low raises the possibility that such complexes result from dissociation of the ~2-MDa complexes during the multiple steps of ion-exchange chromatography used in their purification (13, 20, 31). Purification of murine Mediator over several ion-exchange columns was followed by immunoblotting for the murine homologs of Med7 and Srb7 (13). The 15-subunit complex that was isolated lacks CDK8 and cyclin C and may represent a core of Mediator subunits that resists dissociation during ion-exchange chromatography. PC2 purification was followed by its ability to stimulate activated transcription in a reaction with highly purified GTFs and Pol II (20). A core complex resulting from dissociation of the ~2-MDa complexes and similar to the murine Mediator may have been selected during isolation because it lacks the CDK8 that inhibits transcription in the purified system but retains subunits required to stimulate activated transcription.

Quantitative immunoblotting of dilutions of whole-cell extract, nuclear extract, and Superose 6 fractions, using purified, recombinant hSur2 of known concentration as a standard (Fig. 2), indicated that there are ~300,000 molecules of hSur2 associated with the ~2-MDa Mediator complexes in HeLa cells. This probably represents the number of Mediator complexes per cell, assuming a stoichiometry of 1 hSur2 polypeptide per complex. This number is equivalent to the numbers of Pol II and GTF molecules per HeLa cell (110,000 to 360,000) measured by similar methods (15). Since Mediator is generally required for most Pol II transcription in yeast (11, 34) and since murine *SRB7*, like yeast *SRB7*, is an essential gene (37), it is likely that mammalian Mediator complexes are required for most Pol II transcription in mammalian cells as well. Consequently, it seems reasonable that the in vivo concentration of Mediator complexes is similar to that of the generally required GTFs. We estimate that the concentration of ~500-kDa Mediator complexes, if they exist separate from the ~2-MDa complexes, is less than 1/10 the concentration of the ~2-MDa

complexes. If this is the case, they are present at much lower concentrations than are the GTFs. However, this would still be equivalent to a large fraction of the active promoters in a HeLa cell (probably <50,000). Consequently, it is possible that a functionally significant number of ~500-kDa Mediator complexes do exist but that they are present at too low a concentration to be evident in the immunoblots of fractionated nuclear extract (Fig. 1).

Most yeast Mediator in whole-cell and nuclear extracts is associated with Pol II (9, 10, 14, 16–18, 38). Liu et al. (18) found that a yeast Pol II-Mediator complex isolated in buffer containing 0.3 M potassium acetate dissociated into separable Mediator and core Pol II in 0.5 M potassium acetate. Mammalian Pol II holoenzyme complexes also have been described that contain Pol II, Mediator subunits, and additional proteins (5, 19, 26–28). However, in our analysis of HeLa nuclear extract prepared in 0.3 M NaCl and subjected to gel filtration in 0.3 M KCl, we did not observe a peak of Pol II at the position of the ~2-MDa Mediator. Most Pol II eluted at ~600 kDa, the position we observed for highly purified core Pol II (data not shown) and at the same position as in a buffer with 1 M KCl (Fig. 1). In long exposures of the immunoblots, Pol II in the leading edge of the ~600-kDa peak could be detected in column fractions containing the Mediator peak, but we did not observe even a small peak of Pol II at this position. We cannot rule out that a small fraction of Pol II is associated with human Mediator in the Dignam et al. (6) nuclear extract. However, since there are similar numbers of Pol II molecules and Mediator complexes in the extract, Pol II cannot be a component of most of the Mediator complexes observed under these conditions. Naar et al. (25) came to a similar conclusion when they found that a low level of Pol II in their purified ARC complex could be immunodepleted without depleting most of the ARC subunits or transcriptional activity. Also, Chiba et al. (4) reported that Pol II is not a stoichimetric component of the DRIP complex. Of considerable interest, they found that when the DRIP complex associated with a nuclear receptor, it was able to bind Pol II. We also observed that the vast majority of the p300 coactivator separated from the Mediator complex during gel filtration in 0.3 M KCl (Fig. 1). Rachez et al. (30) similarly observed that the closely related CBP coactivator is readily separated from the DRIP complex by velocity sedimentation in a glycerol gradient run in a low-salt buffer. Why do we and others observe ~2-MDa Mediator complexes that are not associated with Pol II, CBP/P300, or general transcription factors while others have detected mammalian holoenzyme complexes containing Mediator subunits, CBP/P300, and general transcription factors (19, 26–28)? It is possible that the methods we used dissociated higher-order holoenzyme complexes that may exist in the cell. The Mediator complexes we observed are stable multisubunit complexes quite resistant to dissociation by high salt concentration, since we observed them eluting from the Superose 6 gel filtration column at the same position in buffers containing 0.3 M and 1 M KCl (Fig. 1). However, as discussed above, these Mediator complexes may dissociate during chromatography on ion-exchange columns to generate the ~500-kDa Mediator complexes that have been described.

Mediator complexes greatly stimulate activated in vitro transcription (1, 2, 8, 20, 25, 30, 31). The results in Fig. 7 show that when HeLa cell nuclear proteins are fractionated by size under conditions chosen to minimize dissociation of multiprotein complexes, this activity is associated with ~2-MDa Mediator complexes. Further studies of these complexes should extend our understanding of the molecular mechanisms regulating transcription in mammalian cells.

ACKNOWLEDGMENTS

This work was supported by NIH grant CA25235. G.T.C. was supported by USPHS NRSA GM07185.

We thank Leonard Freedman, Emma Lees, and Danny Reinberg for antisera to DRIP150, CDK8 and cyclin C, and hMed6, respectively, and we thank Carol Eng for excellent technical assistance. We also thank Leonard Freedman for instructing us in the method of DRIP complex purification by binding to Gst-VDR(LBD) followed by peptide elution, and we thank Liangru Shi at BD Pharmingen for collaborating in the isolation of an anti-hSur2 monoclonal antibody.

REFERENCES

1. **Akoulitchev, S., S. Chuikov, and D. Reinberg.** 2000. TFIIH is negatively regulated by cdk8-containing mediator complexes. Nature **407**:102–106.
2. **Boyer, T. G., M. E. Martin, E. Lees, R. P. Ricciardi, and A. J. Berk.** 1999. Mammalian Srb/Mediator complex is targeted by adenovirus E1A protein. Nature **399**:276–279.
3. **Carlson, M.** 1999. Glucose repression in yeast. Curr. Opin. Microbiol. **2**:202–207.
4. **Chiba, N., Z. Suldan, L. P. Freedman, and J. D. Parvin.** 2000. Binding of liganded vitamin D receptor to the vitamin D receptor interacting protein coactivator complex induces interaction with RNA polymerase II holoenzyme. J. Biol. Chem. **275**:10719–10722.
5. **Cho, H., G. Orphanides, X. Sun, X. J. Yang, V. Ogryzko, E. Lees, Y. Nakatani, and D. Reinberg.** 1998. A human RNA polymerase II complex containing factors that modify chromatin structure. Mol. Cell. Biol. **18**:5355–5363.
6. **Dignam, J. D., P. L. Martin, B. S. Shastry, and R. G. Roeder.** 1983. Eukaryotic gene transcription with purified components. Methods Enzymol. **101**: 582–598.
7. **Fondell, J. D., H. Ge, and R. G. Roeder.** 1996. Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. Proc. Natl. Acad. Sci. USA **93**:8329–8333.
8. **Gu, W., S. Malik, M. Ito, C. X. Yuan, J. D. Fondell, X. Zhang, E. Martinez, J. Qin, and R. G. Roeder.** 1999. A novel human SRB/MED-containing cofactor complex, SMCC, involved in transcription regulation. Mol. Cell **3**:97–108. (Erratum, **3**:541.)
9. **Gustafsson, C. M., L. C. Myers, J. Beve, H. Spahr, M. Lui, H. Erdjument-Bromage, P. Tempst, and R. D. Kornberg.** 1998. Identification of new mediator subunits in the RNA polymerase II holoenzyme from *Saccharomyces cerevisiae*. J. Biol. Chem. **273**:30851–30854.
10. **Han, S. J., Y. C. Lee, B. S. Gim, G. H. Ryu, S. J. Park, W. S. Lane, and Y. J. Kim.** 1999. Activator-specific requirement of yeast mediator proteins for RNA polymerase II transcriptional activation. Mol. Cell. Biol. **19**:979–988.
11. **Holstege, F. C., E. G. Jennings, J. J. Wyrick, T. I. Lee, C. J. Hengartner, M. R. Green, T. R. Golub, E. S. Lander, and R. A. Young.** 1998. Dissecting the regulatory circuitry of a eukaryotic genome. Cell **95**:717–728.
12. **Ito, M., C. X. Yuan, S. Malik, W. Gu, J. D. Fondell, S. Yamamura, Z. Y. Fu, X. Zhang, J. Qin, and R. G. Roeder.** 1999. Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. Mol. Cell **3**:361–370.
13. **Jiang, Y. W., P. Veschambre, H. Erdjument-Bromage, P. Tempst, J. W. Conaway, R. C. Conaway, and R. D. Kornberg.** 1998. Mammalian mediator of transcriptional regulation and its possible role as an end-point of signal transduction pathways. Proc. Natl. Acad. Sci. USA **95**:8538–8543.
14. **Kim, Y. J., S. Bjorklund, Y. Li, M. H. Sayre, and R. D. Kornberg.** 1994. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell **77**:599–608.
15. **Kimura, H., Y. Tao, R. G. Roeder, and P. R. Cook.** 1999. Quantitation of RNA polymerase II and its transcription factors in an HeLa cell: little soluble holoenzyme but significant amounts of polymerases attached to the nuclear substructure. Mol. Cell. Biol. **19**:5383–5392.
16. **Koleske, A. J., and R. A. Young.** 1994. An RNA polymerase II holoenzyme responsive to activators. Nature **368**:466–469.
17. **Liao, S. M., J. Zhang, D. A. Jeffery, A. J. Koleske, C. M. Thompson, D. M. Chao, M. Viljoen, H. J. van Vuuren, and R. A. Young.** 1995. A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature **374**:193–196.
18. **Liu, Y., J. A. Ranish, R. Aebersold, and S. Hahn.** 2001. Yeast nuclear extract contains two major forms of RNA polymerase II mediator complexes. J. Biol. Chem. **276**:7169–7175.
19. **Maldonado, E., R. Shiekhattar, M. Sheldon, H. Cho, R. Drapkin, P. Rickert,**

E. Lees, C. W. Anderson, S. Linn, and D. Reinberg. 1996. A human RNA polymerase II complex associated with SRB and DNA-repair proteins. Nature 381:86–89. (Erratum, 384:384, 1996.)
20. Malik, S., W. Gu, W. Wu, J. Qin, and R. G. Roeder. 2000. The USA-derived transcriptional coactivator PC2 is a submodule of TRAP/SMCC and acts synergistically with other PCs. Mol. Cell 5:753–760.
21. Malik, S., and R. G. Roeder. 2000. Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. Trends Biochem. Sci. 25:277–283.
22. Martin, M. E., and A. J. Berk. 1999. Corepressor required for adenovirus E1B 55,000-molecular-weight protein repression of basal transcription. Mol. Cell. Biol. 19:3403–3414.
23. Myer, V. E., and R. A. Young. 1998. RNA polymerase II holoenzymes and subcomplexes. J. Biol. Chem. 273:27757–27760.
24. Myers, L. C., C. M. Gustafsson, D. A. Bushnell, M. Lui, H. Erdjument-Bromage, P. Tempst, and R. D. Kornberg. 1998. The Med proteins of yeast and their function through the RNA polymerase II carboxy-terminal domain. Genes Dev. 12:45–54.
25. Naar, A. M., P. A. Beaurang, S. Zhou, S. Abraham, W. Solomon, and R. Tjian. 1999. Composite co-activator ARC mediates chromatin-directed transcriptional activation. Nature 398:828–832.
26. Neish, A. S., S. F. Anderson, B. P. Schlegel, W. Wei, and J. D. Parvin. 1998. Factors associated with the mammalian RNA polymerase II holoenzyme. Nucleic Acids Res. 26:847–853.
27. Ossipow, V., J. P. Tassan, E. A. Nigg, and U. Schibler. 1995. A mammalian RNA polymerase II holoenzyme containing all components required for promoter-specific transcription initiation. Cell 83:137–146.
28. Pan, G., T. Aso, and J. Greenblatt. 1997. Interaction of elongation factors TFIIS and elongin A with a human RNA polymerase II holoenzyme capable of promoter-specific initiation and responsive to transcriptional activators. J. Biol. Chem. 272:24563–24571.
29. Rachez, C., M. Gamble, C. P. Chang, G. B. Atkins, M. A. Lazar, and L. P. Freedman. 2000. The DRIP complex and SRC-1/p160 coactivators share similar nuclear receptor binding determinants but constitute functionally distinct complexes. Mol. Cell. Biol. 20:2718–2726.
30. Rachez, C., B. D. Lemon, Z. Suldan, V. Bromleigh, M. Gamble, A. M. Naar, H. Erdjument-Bromage, P. Tempst, and L. P. Freedman. 1999. Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. Nature 398:824–828.
31. Ryu, S., S. Zhou, A. G. Ladurner, and R. Tjian. 1999. The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1. Nature 397:446–450.
32. Sun, X., Y. Zhang, H. Cho, P. Rickert, E. Lees, W. Lane, and D. Reinberg. 1998. NAT, a human complex containing Srb polypeptides that functions as a negative regulator of activated transcription. Mol. Cell 2:213–222.
33. Tantin, D., T. Chi, R. Hori, S. Pyo, and M. Carey. 1996. Biochemical mechanism of transcriptional activation by GAL4-VP16. Methods Enzymol. 274: 133–149.
34. Thompson, C. M., and R. A. Young. 1995. General requirement for RNA polymerase II holoenzymes in vivo. Proc. Natl. Acad. Sci. USA 92:4587–4590.
35. Thompson, N. E., D. B. Aronson, and R. R. Burgess. 1990. Purification of eukaryotic RNA polymerase II by immunoaffinity chromatography. Elution of active enzyme with protein stabilizing agents from a polyol-responsive monoclonal antibody. J. Biol. Chem. 265:7069–7077.
36. Tong, J. K., C. A. Hassig, G. R. Schnitzler, R. E. Kingston, and S. L. Schreiber. 1998. Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. Nature 395:917–921.
37. Tudor, M., P. J. Murray, C. Onufryk, R. Jaenisch, and R. A. Young. 1999. Ubiquitous expression and embryonic requirement for RNA polymerase II coactivator subunit Srb7 in mice. Genes Dev. 13:2365–2368.
38. Wilson, C. J., D. M. Chao, A. N. Imbalzano, G. R. Schnitzler, R. E. Kingston, and R. A. Young. 1996. RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling. Cell 84:235–244.
39. Xue, Y., J. Wong, G. T. Moreno, M. K. Young, J. Cote, and W. Wang. 1998. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol. Cell 2:851–861.
40. Zhang, Y., H. H. Ng, H. Erdjument-Bromage, P. Tempst, A. Bird, and D. Reinberg. 1999. Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev. 13:1924–1935.
41. Zhang, Y., Z. W. Sun, R. Iratni, H. Erdjument-Bromage, P. Tempst, M. Hampsey, and D. Reinberg. 1998. SAP30, a novel protein conserved between human and yeast, is a component of a histone deacetylase complex. Mol. Cell 1:1021–1031.
42. Zhou, Q., P. M. Lieberman, T. G. Boyer, and A. J. Berk. 1992. Holo-TFIID supports transcriptional stimulation by diverse activators and from a TATA-less promoter. Genes Dev. 6:1964–1974.